ORIGINAL ARTICLE

Molecular and clinical significance of FLT3, NPM1, DNMT3A and TP53 mutations in acute myeloid leukemia patients

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Abstract

Background Acute myeloid leukemia (AML) is a type of blood cancer that affects the bone marrow and blood cells. AML is characterized by the rapid growth and accumulation of abnormal white blood cells, known as myeloblasts, which interfere with the production of normal blood cells.

Aims The main aim was to determine the relationship between these genetic alterations and the clinico-haematological parameters and prognostic factors with therapy for Iraqi patients with AML.

Methods We used Sanger Sequencing to detect the mutations in 76 AML patients. Clinical data of AML patients were retrospectively analysed to compare the prognosis of each gene mutation group.

Results Somatic mutations were identified in 47.4% of the enrolled patients in a core set of pathogenic genes, including *FLT3* (18 patients, 23.7%), *DNMT3A* (14, 18.4%), *NPM1* (11, 14.5%) and *TP53* (5, 6.8%). As multiple mutations frequently coexisted in the same patient, we classified patients into 10 further groups. Two novel mutations were detected in *FLT3-ITD*, with new accession numbers deposited into NCBI GenBank (OP807465 and OP807466). These two novel mutations were computationally analysed and predicted as disease-causing mutations. We found significant differences between patients with and without the detected mutations in disease progression after induction therapy (remission, failure and death; $pv = < 0.001$) and statistically significant differences were reported in total leukocyte count ($pv = < 0.0001$).

Conclusion These genes are among the most frequently mutated genes in AML patients. Understanding the molecular and clinical significance of these mutations is important for guiding treatment decisions and predicting patient outcomes.

Introduction

Acute myeloid leukemia (AML) is a highly aggressive and malignant cancer that affects the blood and bone marrow. It is the most common form of acute leukemia. The World Health Organization (WHO) classifies AML into various subtypes based on genetic and molecular characteristics. AML is characterized by the uncontrolled growth of immature white blood cells called myeloblasts [1]. It is well established that genetic mutations play a critical role in the diagnosis, prognosis and treatment of AML, these mutations are associated with different clinical and biological characteristics of the disease, and they can be used to stratify patients into different risk categories and inform treatment decisions, which is fully reflected in the National Comprehensive Cancer Network (NCCN) guidelines for AML [2]. In de novo acute AML, *FLT3, DNMT3A, NPM1*, and *TP53* mutations are among the most frequent genomic changes. These changes are important for the pathogenesis and progression of the disease, especially when AML-related recurrent cytogenetic abnormalities are absent [3, 4].

The FLT3 receptor, which belongs to the type-III tyrosine kinase family, plays a critical role in cell growth, differentiation, and survival. One of the most frequent mutations in AML is an internal tandem duplication of FLT3's juxtamembrane domain (*FLT3-ITD*) which is located on

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chromosome 13q12 [5]. Approximately 25% of AML cases exhibit this particular feature. It is strongly linked to the disease and is associated with a higher risk of relapse and lower overall survival rates (OS; median 16.2 months) [5, 6]. AML patients with an *FLT3-ITD* mutation frequently have high bone marrow blast cell counts, total leucocyte counts (WBCs), and elevated rates of relapse, suggesting that *FLT3-ITD* mutation warn a particularly poor prognosis of AML, with constitutive activation of the kinase within this variation [7].

Additionally, one of the most common chromosomal changes observed in AML is a mutation in the nucleophosmin (NPM1) gene. This gene is situated on chromosome 5q35 and is responsible for encoding a protein that shuttles between the nucleus and the cytoplasm. NPM1 plays a crucial role in regulating pathways related to cell proliferation and the suppression of growth [8]. The mutation in the NPM1 gene has been classified as a Class II mutation, which impacts the transcription and differentiation processes in hematopoiesis. This mutation is found in approximately 20–30% of freshly diagnosed cases of acute myeloid leukemia (AML). Moreover, it is detected in around 50% of cases where the karyotype (chromosomal structure) is considered normal [4, [8]. NPM1 is a chaperone protein that performs a variety of tasks while moving back and forth between the cytoplasm and nucleus. *NPM1* mutations have been linked to leukaemia via a number of different mechanisms, but despite the growing understanding of these mechanisms, a focused therapy for *NPM1*-mutated AML is still lacking [4].

Moreover, Tumor protein 53 (TP53) is a tumor suppressor gene that codes for a protein that regulates cell growth and division; it plays a crucial role in regulating the cell cycle, DNA repair, and apoptosis (programmed cell death), and is located on chromosome 17p13.1. Mutations in the TP53 gene are found in approximately 5–10% of AML cases. The mutations in the *TP53* gene locus may be point mutations or deletions and either somatic or germline [9]. TP53 mutations can occur as a primary event or as a secondary event during disease progression or treatment. TP53 mutations can result in the loss of *p53* function, leading to the accumulation of DNA damage and genomic instability, which can contribute to leukemogenesis and resistance to chemotherapy [4]. Additionally, mutations or deletions in *p53* are associated with a poor prognosis, as they can lead to chemotherapy resistance and disease relapse. Patients with AML and p53 mutations often have a higher risk of treatment failure and shorter overall survival than those without p53 mutations [10, 11].

However, nearly 50% of AML samples have a normal karyotype, and many patients carry no mutation. In contrast, DNA methylation patterns are altered in numerous cancers and often correlate with clinically relevant information such as subtypes, prognosis and drug response. Indeed, aberrant DNA methylation patterns are a hallmark of AML [2, 12]. The de novo methyl transferase 3 A (DNMT3A) gene encodes a 130-kDa protein that is involved in epigenetic regulation. Downstream genes' expression is decreased as a result of DNMT3A's ability to catalyse the addition of a methyl group to cytosine residues in CpG islands [12]. R882H is the most common mutation, and it has been shown to act as a dominant-negative on wild-type DNMT3A [12].

The Cancer Genome Atlas (TCGA) team recently discovered a subgroup of AML patients in whom *DNMT3A*, *FLT3* and *NPM1* mutations coexist at a higher frequency than would be predicted for a chance occurrence as a result of an integrated genomic study of de novo AML [13]. It is common that multiple variations occurs simultaneously in the same patient, indicating the role of concerted interaction of mutations in the pathogenesis of AML [14]. Advances in genetic testing and sequencing technologies have made it possible to identify and analyze multiple mutations simultaneously, leading to a better understanding of the complex genetic landscape of AML and the development of targeted therapies that can improve outcomes for patients [13].

Treatment for AML is a long-term process that depends on several factors, including the patient's age, overall health, and subtype of AML. The FLT3 status of a patient can be helpful for prognosis and identifying patients who receive treatment with a FLT3 inhibitor, or for alternate consolidations such as stem cell transplants [15]. The most commonly used therapies are chemotherapy and stem cell transplantation; however, the majority of patients die of AML relapse [12].

To enhance outcomes for patients with AML, it is imperative to conduct clinical research investigating new medications and combinations of drugs. Traditionally, drug development efforts have primarily concentrated on conducting dose-finding studies for single-agent therapies in the relapsed setting. This approach has led to the approval of effective targeted treatments, such as FLT3 and DNMT3A inhibitors. By following this approach, researchers aim to identify novel therapies that specifically target the NPM1 mutation, with the goal of improving treatment outcomes for patients with AML who carry this mutation [16, 17]. The fundamentals of intensive chemotherapy continue to be anthracyclines and cytarabine. Alternatives include mitoxantrone-based cytarabine regimens and fludarabine, cytarabine, granulocyte colony-stimulating factor and idarubicin (FLAG-IDA). For patients with FLT3-mutant AML, it has been common practice to include the kinase inhibitor midostaurin (Rydapt) and/or gilteritinib (Xospata) in first-line therapy [16]. Other treatments (i.e. epigenetically targeted therapies) and immunotherapy methods, such as bispecific T-cell engaging antibodies, checkpoint inhibitors,

chimeric antigen receptor T cells, or natural killer cells, are probably most efficient in frontline or early salvage combination approaches when used in the presence of minimal residual disease (MRD) [18]. Hence, the objectives of the present study is to establish the correlation between molecular mutations such as *FLT3-ITD, DNMT3A, NPM1* and *TP53* and clinico-haematological parameters, as well as the prognosis and outcomes of induction therapy in Iraqi AML patients.

Patients, material and methods

Clinical investigations and patients selection

A total of 76 patients with newly diagnosed de novo AML were enrolled at Hiwa Hospital for Cancer in Sulaimania, Iraq, between October 2021 and March 2022. Patients who were on chemotherapy and those who have completed the courses of treatment were excluded. The patients were sourced from various geographic locations within the Iraqi governorates as the hospital serves a wide area. The demographic data captured included age and sex of the patients.

The patients were confirmed to have AML through hematological testing, the diagnosis of AML typically involves the identification and enumeration of blasts based on morphology in the bone marrow (BM) aspirate. This process often requires manual examination. Another laboratory analysis involved a complete blood count (CBC), which included measurements of total WBCs, platelet (Plt) count, and Hemoglobin (Hb) levels. The CBC analysis was performed using a fully automated M-series hematology analyzer called the Medonic CBC Analyzer (manufactured by Boule Medical AB in Stockholm, Sweden). Furthermore, the study included information regarding the outcome of induction therapy for the patients. Induction therapy refers to the initial treatment given to induce remission in AML patients. Reporting the outcome of induction therapy (OIT) provides insights into the effectiveness of the treatment and contributes to understanding the clinical traits of the AML

patients in the study. Table 1 provides an overview of the sociodemographic characteristics and clinical traits of the AML patients in the current study.

Genotyping and targeting genes mutations in AML

DNA extraction

In the study, genomic DNA was extracted from EDTA blood samples in a biosafety level 2 laboratory using the Qiagen QIAamp DNA Blood Kit, manufactured in Hilden, Germany. The extraction procedure followed the manufacturer's recommended protocol.

To assess the concentration and purity of the extracted DNA, the ratio of optical density at 260/280 nm was measured using a NanoDrop spectrophotometer from Thermo Fisher Scientific, located in Waltham, MA, USA. This ratio provides an indication of DNA concentration and purity, with values around 1.8-2.0 considered optimal. The extracted DNA samples were then frozen at -20 °C until they were needed for further analysis or experimentation.

Polymerase chain reaction (PCR)

For PCR amplification of selected genes, primers were designed using the Primer3 + [19] and NCBI Primer-Blast web-based tools [20]. During primer design, criteria such as a higher melting temperature (Tm) were considered to ensure optimal primer annealing and specificity. Hence, we then selected those primers that have a higher melting temperature (Tm) and can specifically and sensitively amplify these regions. The PCR reaction was carried out using a total volume of 20 µl, consisting of 3 µl DNA template, 10 µl nuclease-free water, 1 µl each of 10 µM forward and reverse primers, 2 µl of a 10 mM dNTPs mixture, 2 µl of a 10X reaction buffer, 0.4 µl of 2.5 U/µl Taq DNA polymerase, and 0.6 µl of MgCl2 from AddBio in Daejeon, South Korea. The thermal cycling program included the following steps: initial melting at 94 °C and the other steps for specific PCR condition for each gene were applied as outlined in Table 2.

Table 1 Summary of primer sequences with PCR protocol optimised conditions for all 4 genes

Gene	Exons	Primer Sequence(5' − 3')	FS.(bp)	PCR conditions
FLT3 – ITD	14–15	GCAATTTAGGTATGAAAGCCAGC CTTTCAGCATTTTGACGGCAACC	329	94°C 7 min. 94°C 35 sec 59°C 50 sec x35. 72°C 2 min 72°C 10 min
NPM1	12	TGACTGATTAAAGTGTTTGGAATTAAA CAAGACTATTTGCCATTCCTAAC	660	94°C 7 min. 94°C 35 sec 58°C 50 sec x35. 72°C 2 min 72°C 10 min
DNMT3A [25]	23	GGCCAGCACTCACCCTGCCC GCCCTCCGGTATTTCCGCCTC	348	94°C 5 min. 94°C 30 sec 56°C 50 sec x45. 72°C 45 s 72°C 10 min
TP53	5–6	TTCCTCTTCCTGCAGTACTC AGTTGCAAACCAGACCTCAG	408	94°C 7 min. 94°C 35 sec 56°C 50 sec x30. 72°C 2 min 72°C 10 min
TP53	7–8	AGGTTGGCTCTGACTGTACC ATTGTCCTGCTTGCTTACCTC	610	94°C 7 min. 94°C 35 sec 56°C 50 sec x30. 72°C 2 min 72°C 10 min

Table 2 Demographic, laboratory and clinical Characteristics with data for patients in the study group

Parameters		Unmutated group (n = 40)	Mutated group (n = 36)	P-value (chi-square)
Age:	15–29	7 (17.5)	3 (8.3)	0.799
	30–49	9 (22.5)	17 (47.3)	
	≥ 50	24 (60.0)	16 (44.4)	
Sex:	Male	18 (45.0)	17 (42.2)	0.848
	Female	22 (55.0)	19 (52.8)	
WBC:	< 10,000	4 (10.0)	2 (5.5)	< 0.0001
$(10*^9/L)$:	10^3–10^5	34 (85.0)	24 (66.7)	
	> 100,000	2 (5.0)	10 (27.8)	
Haemoglobin:	< 10	7 (17.5)	3 (8.3)	0.523
(d/dL)	≥ 10	33 (82.5)	33 (91.7)	
Platelets:	< 100	35 (87.5)	30 (83.3)	0.351
$(10*^9/L)$	100–400	5 (12.5)	5 (13.9)	
	> 400	0 (0.0)	1 (2.8)	
Blasts (%):	< 5%	0 (0.0)	0 (0.0)	0.319
	5–50%	25 (62.5)	14 (38.9)	
	> 51%	15 (37.5)	22 (61.1)	
Outcome on day 60:				< 0.001
Complete remission		34 (85.0)	26 (72.2)	
Failed remission		4 (10.0)	7 (19.5)	
Death		2 (5.0)	3 (8.3)	

Note: Data presented as number (n) and percentage (%). Chi-square significant at p ≤ .05. p-Value style: GP: >0.05 (ns)

The PCR products were visualized using 2.5% agarose gels stained with SYBR Safe dye from Thermo Fisher Scientific. The gels were examined under ultraviolet light at wavelengths of 329, 660, 348, 408, and 610 nm (Table 2 and supplementary Fig. 1).

In addition to the patient group, a control group comprising 25 volunteers was included. The selection of individuals for the control group was based on a questionnaire, ensuring that they were from families with no reported cases or history of cancer. The control group was matched with the patient group based on age and gender to account for potential confounding factors. Hence, we aimed to identify the most common genes that are mutated in AML and determine the regions of high mutation within those genes such as FLT3-ITD [21]. DNMT3A [22], NPM1 [23] gene and TP53 [24] gene.

Sanger sequencing and computational databases

To perform Sanger sequencing, the PCR products obtained for each targeted amplicon were purified using the AddPrep PCR Purification Kit from AddBio. Primers specifically designed for this study were used in the purification process. The purified products were then subjected to nucleotide sequencing using the Sanger sequencing method, which was conducted at a sequencing facility located in South Korea [26].

Following sequencing, the obtained nucleotide sequences were analyzed using the computational FinchTV v.1.4 software (Geospiza, Inc. http://www.geospiza.com/finchtv). This software allowed for manual alignment, editing, and justification of each individual sequence to ensure accuracy. Pairwise alignment of the sequences was further validated using the BLAST software tool, which helps confirm the consistency and reliability of the alignments.

Various computational tools were employed to assess the sequences, identify amino acid variants, and determine the novelty of the mutations reported in the study. These tools included the ExPASy ProtParam online tool [27], the NCBI SNP databases (dbSNP), The 1,000 Genomes Project, HGMD Professional software, and the Ensemble browser. By utilizing these resources, researchers could gather comprehensive information regarding the sequence variations, potential effects on amino acids, and the presence of known mutations or variations reported in existing databases and projects [25]. The relevant accession numbers were obtained from the NCBI GenBank databases.

Statistical analysis

In the analysis of the data, the GraphPad Prism 9 software was used to perform a Two-Way Analysis of Variance (ANOVA). This statistical analysis was employed to assess the differences in the investigated parameters between the two groups: mutated AML cases, unmutated AML cases, and normal cases. The ANOVA helps determine if there are significant variations in the measured parameters among these groups.

To explore the relationships between various factors and the OIT, Pearson correlation and polynomial regression analyses were conducted. These analyses aimed to examine the correlations among gender, age, location, total WBCs, Hb levels, PLT, peripheral blasts, and the OIT. To investigate the relationships between the two groups (AML cases and the control group), as well as between the OIT and patient demographic parameters, Pearson's chi-square test was used. This statistical test examines the association between categorical variables. The test was employed to determine if there were significant relationships between the groups and the OIT based on the patient demographic parameters. In all statistical analyses, a significance level of $p < .05$ was used, indicating that results with p-values below this threshold were considered statistically significant.

Results

Study group characteristics

A total of 76 AML cases were included in the study, with 56.2% being male and 43.8% female. The age of the patients ranged from 18 to 89 years, with a mean age of 50 years. The most common age observed among the patients was 59 years, as evidenced by a peak in the age distribution.

Table 1 provides information on the age, gender, WBCs, Hb levels, Plt count, blast percentage, and outcome of remission for all patients.

Importantly, no mutations were detected in any of the 25 control cases. The frequency of germline mutations differed significantly between the tested group (47.4%) and the control group (0%), with a statistically significant p-value of 0.001. This suggests a notable difference in the occurrence of mutations between the AML cases and the control group, emphasizing the relevance of these mutations in AML development.

Molecular landscape and mutation analysis

Frequencies of gene mutations

As shown in Fig. 3, the frequencies for all four genes selected in this study were *FLT3*: 18 patients (23.7%); *DNMT3A*: 14 patients (18.4%) [25]; *NPM1*: 11 patients (14.5%) and *TP53*: 5 patients (6.8%), Fig. 1A. Using combined genetic markers, the AML patients were further classified into 10 groups. The largest group, group 1, accounted for 52.6% of the patients and comprised those without any detected

mutations. The other groups were categorized based on specific mutations.

The distribution of patients in the mutation-specific groups is as follows: group 2 consisted of patients with FLT3-ITD mutations only (14.5%); group 3 had DNMT3A mutations only (9.2%); group 4 contained NPM1 mutations only (6.7%); group 5 had TP53 mutations only (5.2%); group 6 comprised patients with FLT3/DNMT3A/NPM1 mutations (4.0%); group 7 consisted of patients with FLT3-ITD/DNMT3A mutations (2.6%); group 8 had FLT3-ITD/NPM1 mutations (2.6%); group 9 contained DNMT3A/NPM1 mutations (1.3%); and group 10 consisted of patients with TP53/DNMT3A mutations (1.3%).

These groups were determined based on the presence or absence of specific mutations, allowing for a more detailed characterization of the genetic profile of AML patients. Figure 1B provides a visual representation of the distribution of patients across these groups.

Detection of FLT3-ITD mutation

We found the incidence of *FLT3-ITD* mutations to be 18 (23.7% of patients). The first *FLT3-ITD* mutation found in this study was c.1784G > T (R595I) and reported in 8 patients: 44.5%, as previously reported [28] (Fig. 2: A and D, M.1), while the other two mutations are reported here for the first time, and were deposited in NCBI-GenBank as novel mutations with a new accession numbers OP807465 (B:c.1820G > C & c.1831G > C: R607T & E611Q: Fig. 2:B and D, M.2; 4 patients: 22.2%) and OP807466 (C:c.1898 A > G: K634E: D,M.3; 6 patients:33.3%).

In this study, computational analysis was performed to assess the potential impact of the two novel mutations on
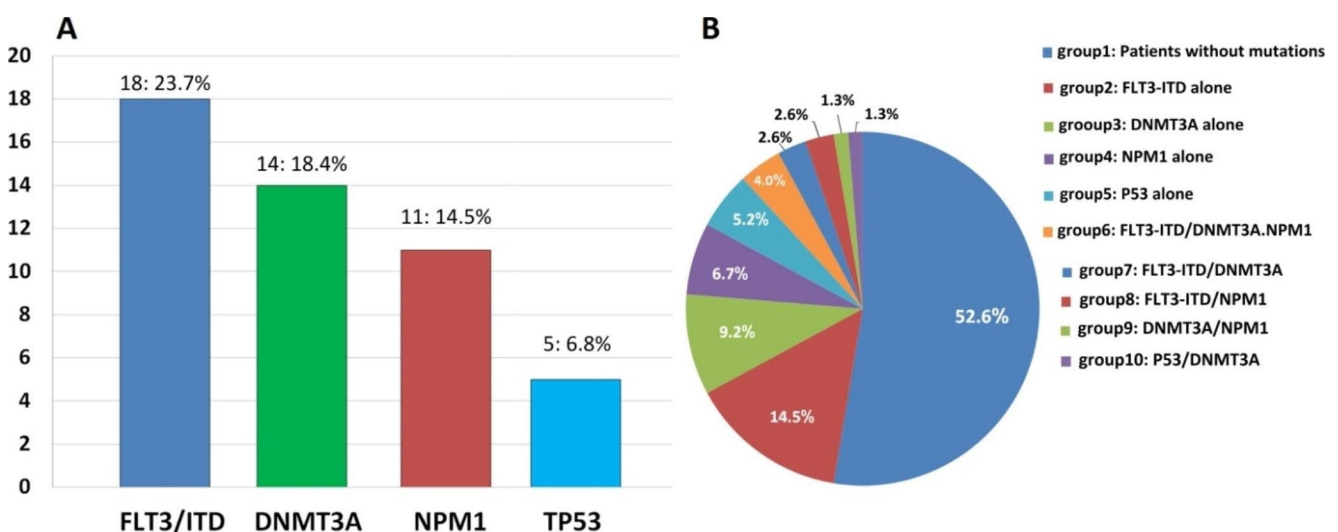

Fig. 1 Frequencies and Distribution of subtypes in AML patients **(A)** Estimated *FLT3*, *DNMT3A*, *NPM1* and *TP53* variant (allelic) frequencies in a subset of de novo acute myeloid leukaemia samples.

(B) Common molecular gene mutation subgroups of de novo acute myeloid leukaemia included in the study
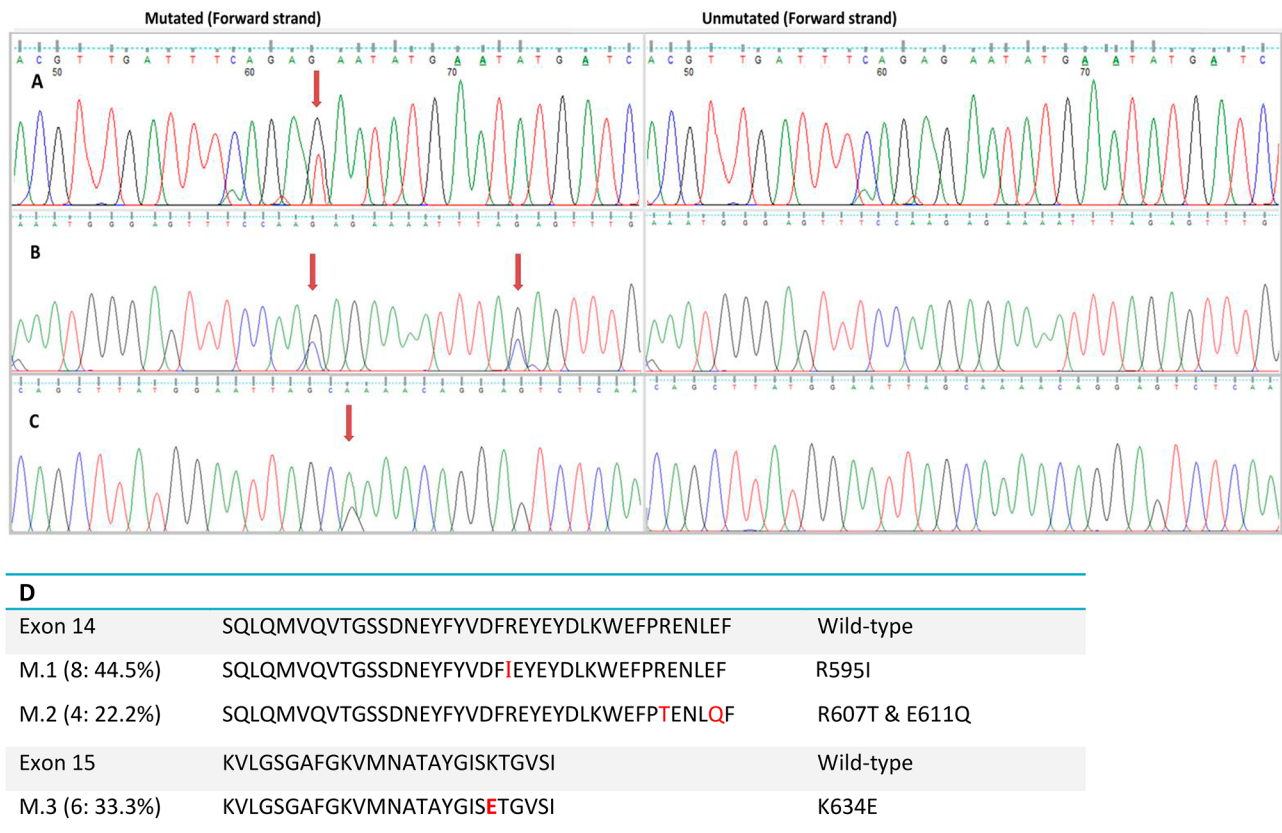
D		
Exon 14	SQLQMVQVTGSSDNEYFYVDFREYEYDLKWEFPRENLEF	Wild-type
M.1 (8: 44.5%)	SQLQMVQVTGSSDNEYFYVDF**I**EYEYDLKWEFPRENLEF	R595I
M.2 (4: 22.2%)	SQLQMVQVTGSSDNEYFYVDFREYEYDLKWEFP**T**ENL**Q**F	R607T & E611Q
Exon 15	KVLGSGAFGKVMNATAYGISKTGVSI	Wild-type
M.3 (6: 33.3%)	KVLGSGAFGKVMNATAYGIS**E**TGVSI	K634E

Fig. 2 Sequencing results of the *FLT3-ITD* mutations and alignment of multiple FLT3-ITD protein sequences. **(A)** Unmutated: The sequence chromatogram of a wild-type allele with Arginine (AGA) at codon 593: Mutated: the mutant allele with a heterozygous mutation of Arg593Iso (c.1783G > T) in affected members. **(B)** Unmutated: The sequence chromatogram of a wild-type allele with Arginine (AGA) at codon 607 and Glutamic acid (GAG) at codon 611: Mutated: the mutant allele with a heterozygous mutation of Arg607Thr (c.1820G > C) and Glu611Gln (c.1831G > C) in affected members. **(C)** Unmutated: The sequence chromatogram of a wild-type allele with Lysine (AAA) at codon 634: Mutated: the mutant allele with a heterozygous mutation of Lys634Glu (c.1898 A > G) in affected members **(D)** protein sequence alignments illustrating three different FLT3-ITD mutations along with their corresponding frequencies

protein stability. The I-mutant tool predicted that the two novel mutations (B: -0.60 and C: -0.70) would decrease protein stability with a Delta Delta G (DDG) value < 0. The VarSite [29] analysis predicted that the mutations (B: 1.15 and C: 1.50) would have a higher probability of being pathogenic than those with values ≤ 1, with a DDG value > 1 [25]. The sequences were aligned using a reliable alignment algorithm to identify conserved and variant regions within the FLT3-ITD mutation Fig. 2D.

Detection of DNMT3A mutation

In our previous study [25], we conducted an examination of a cohort that consisted of both the same patient sample as well as an additional fifteen AML patients with negative *DNMT3A* detected mutations, as confirmed in the current study. Within the context of our previous research, we made an intriguing discovery concerning three previously novel DNMT3A mutations which have been deposited in the

GenBank NCBI for public access (Data availability). These mutations were observed in 14 out of the total 76 patients, accounting for approximately 18.4% of the patient population. These findings highlight the significance of these novel DNMT3A mutations and suggest their potential involvement in the pathogenesis of AML.

Detection of NPM1 mutation

In this study, eleven patients were found to have two different *NPM1* mutations (C860-863dupTCTG and C868G > T), accounting for 14.5% of all the currently enrolled patients (Fig. 3A mutation A and B: A.mut). The first mutation found in exon 12 (the most frequently detected mutation in *NPM1*) [8] was C860-863dupTCTG, accounting for 72.2% of *NPM1* mutations, as reported in previous study [30]. The second mutation, c868G > T also in exon 12 accounted for 27.3% of the *NPM1* mutations, as reported earlier in Iraq [31] (Fig. 3A mutation x and B: B.mut).
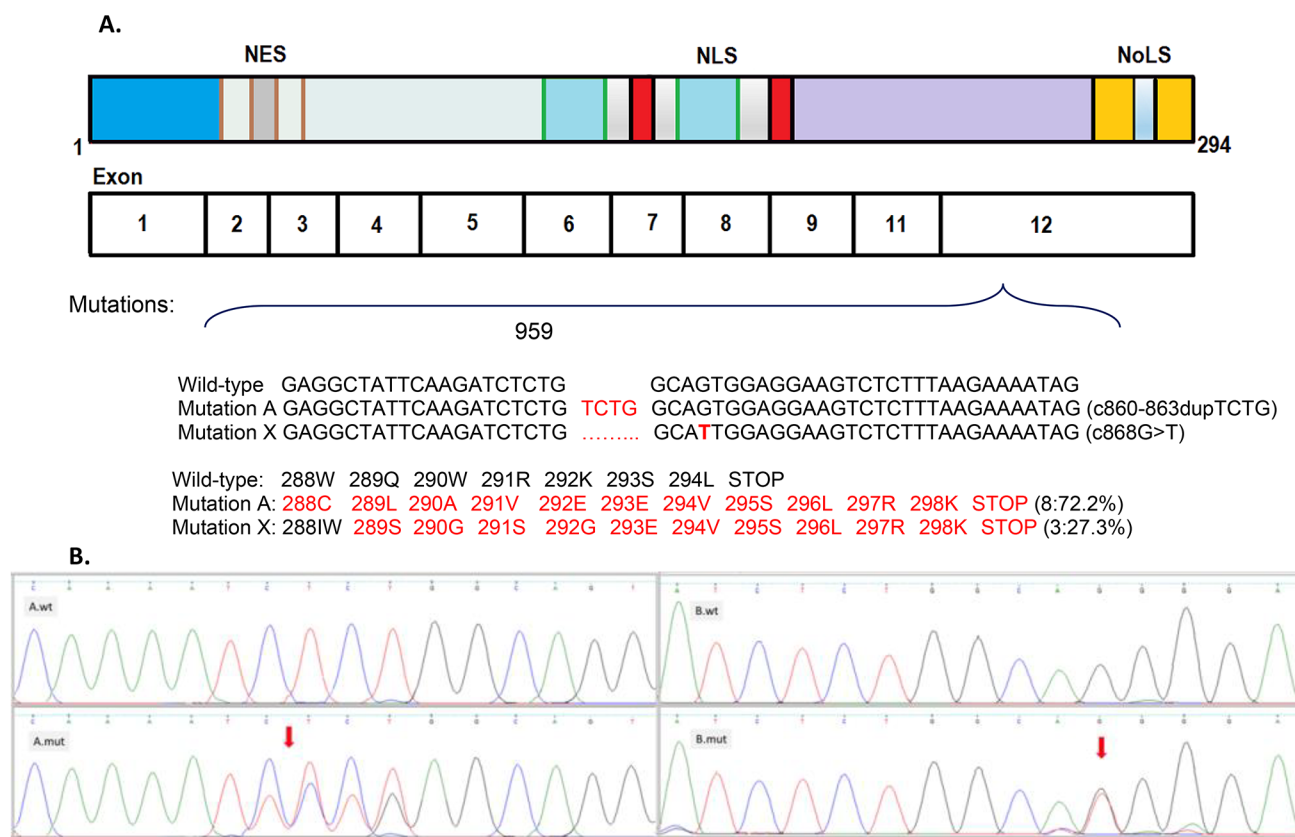
Fig. 3 The gene structure of *NPM1* along with the mutations associated with AML. **(A).** changes of tryptophans (288 and 290) and insertion of a new nuclear export signal (NES) motif. These changes cause aberrant cytoplasmic accumulation in *NPM1* mutants. **(B).** The chromatogram shows the representative sequencing results of wild-type and mutant patients

Detection of TP53 mutation

In present study, we detected two types of p53 mutations in 5 of 76 (6.9%) patients with AML (Fig. 4A & B): The first one is I225N substitution caused by the heterozygous c.764T > A, and the next one is A276P substitution that caused by the heterozygous c.826G > C. Importantly, these p53 mutations detected in our study have previously been reported in AML patients, indicating their relevance and significance in the context of AML pathogenesis [32, 33]. Figure 4 provides a visual representation that aids in understanding the specific alterations occurring within the TP53 gene at the protein level. The aligned sequences allow for a comparison between the wild-type TP53 sequence and the mutated variants, highlighting the specific sequence and amino acid changes associated with each mutation in the current study.

The epidemiological profile

Figure 5 provides a summary of the clinical characteristics of the AML patients (mutated and unmutated) compared to the control group in this study. The analysis examined sociodemographic data such as gender, age, and location, as well as various clinical parameters including total WBCs, PLT, Hb, and peripheral blast count.

The results indicated that there were no significant differences between the patients (mutated and unmutated) and the control group in terms of gender, age, and location. However, significant differences were observed between the two groups in terms of other clinical parameters, namely total WBCs, platelets, Hb, and peripheral blast count (all with p-values < 0.0001). Furthermore, the mutation cases showed a significant difference in total WBCs compared to the unmutated cases. A t-test analysis revealed that WBCs were significantly higher in mutated cases compared to unmutated cases (mean difference: -33.36 ± 6.668, p-value < 0.0001).

In contrast, the comparisons of other epidemiological profiles showed only slight differences between the means, without significant data reported for gender (mean difference: 0.02222 ± 0.1160, p-value = 0.8486), age (mean difference: -0.8667 ± 3.403 years, p-value = 0.7997), location (mean difference: 0.06667 ± 0.1120, p-value = 0.5536), platelets
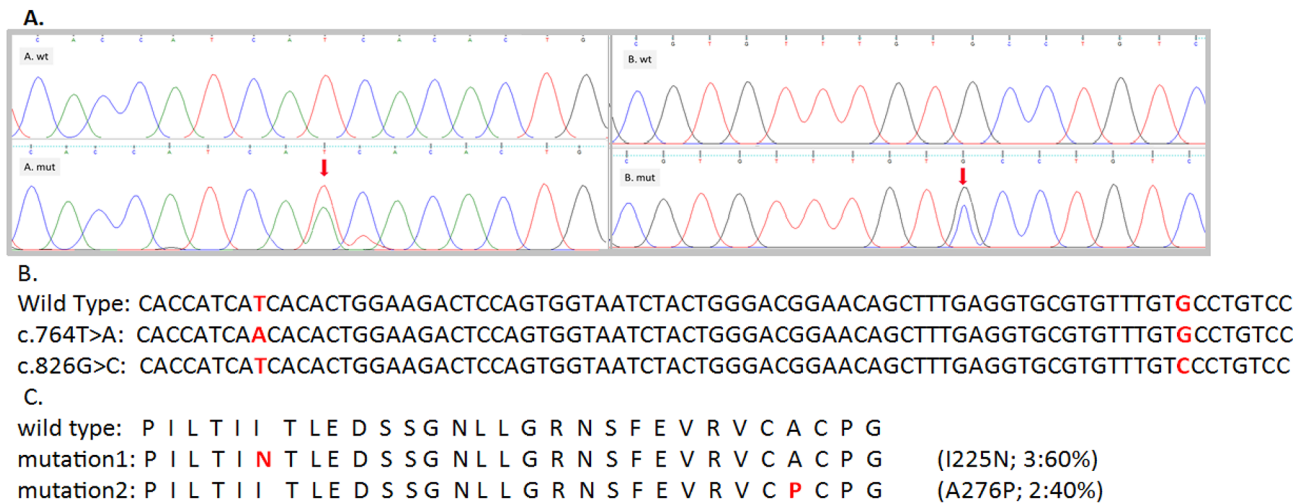
Fig. 4 Patterns of TP53 mutations: **(A)** the chromatogram shows the representative sequencing results of wild type and mutant patients **(B)** Sequences for wild-type and the two detected mutations c.764T>A and c.826G>C **(C)** Protein sequence alignments illustrating two different TP53 mutations along with their corresponding frequencies

(mean difference: -30.42 ± 32.46, p-value = 0.3518), and blasts (mean difference: -4.736 ± 4.724, p-value = 0.3194).

Hence, the analysis revealed significant differences in certain clinical parameters between the mutated and unmutated AML cases, particularly in total WBCs, while no significant differences were observed in gender, age, location, platelets, and blasts.

The analysis conducted on the epidemiological profile using Pearson correlation and polynomial regression revealed several correlations among the variables. In Fig. 6, the red boxes represent variables with a negative relationship, while the blue boxes represent variables with a positive relationship.

In AML mutated and unmutated patients, the total WBC count exhibited a highly positive correlation with FLT3-ITD and DNMT3A mutations, with correlation coefficients of 0.48 and 0.35, respectively. This indicates that as the total WBC count increases, the likelihood of having FLT3-ITD and DNMT3A mutations also increases. The outcome of induction therapy showed significant correlations as well, particularly in patients with DNMT3A mutations, with a correlation coefficient of 0.48 (Fig. 6A). This suggests that patients with DNMT3A mutations had similar outcomes in induction therapy.

Furthermore, in AML patients compared to the control group, there were high positive correlations observed, particularly between WBCs and variables such as overall induction therapy (OIT) (correlation coefficient of 0.60), FLT3 mutation (correlation coefficient of 0.53), and blast count (correlation coefficient of 0.56). Additionally, correlations were observed between DNMT3A mutation and OIT (correlation coefficient of 0.50) and between blast count and OIT (correlation coefficient of 0.64) (Fig. 6B). These findings indicate that there is a strong positive relationship between these variables in AML patients compared to the control group.

Overall, these results highlight the associations between various variables in AML patients, including the total WBC count, specific gene mutations (FLT3-ITD and DNMT3A), and the outcome of induction therapy. The correlations found in the study provide insights into the relationships between these factors, which can contribute to a better understanding of AML and potentially aid in its management and treatment.

Treatment response analysis and follow up

In the treatment of AML, the therapeutic process can be lengthy, typically taking 6 to 12 months to complete, which includes chemotherapy and other treatment modalities. In this study, we considered two different outcomes based on the presence or absence of the investigated mutations.

Among the 76 AML patients with detected mutations, the outcomes of 60 days after complete treatment with induction therapy were as follows: 26 (72.2%) achieved remission, 7 (19.5%) experienced treatment failure and 3 (8.3%) unfortunately passed away. On the other hand, for the patients without the detected mutations, the outcomes
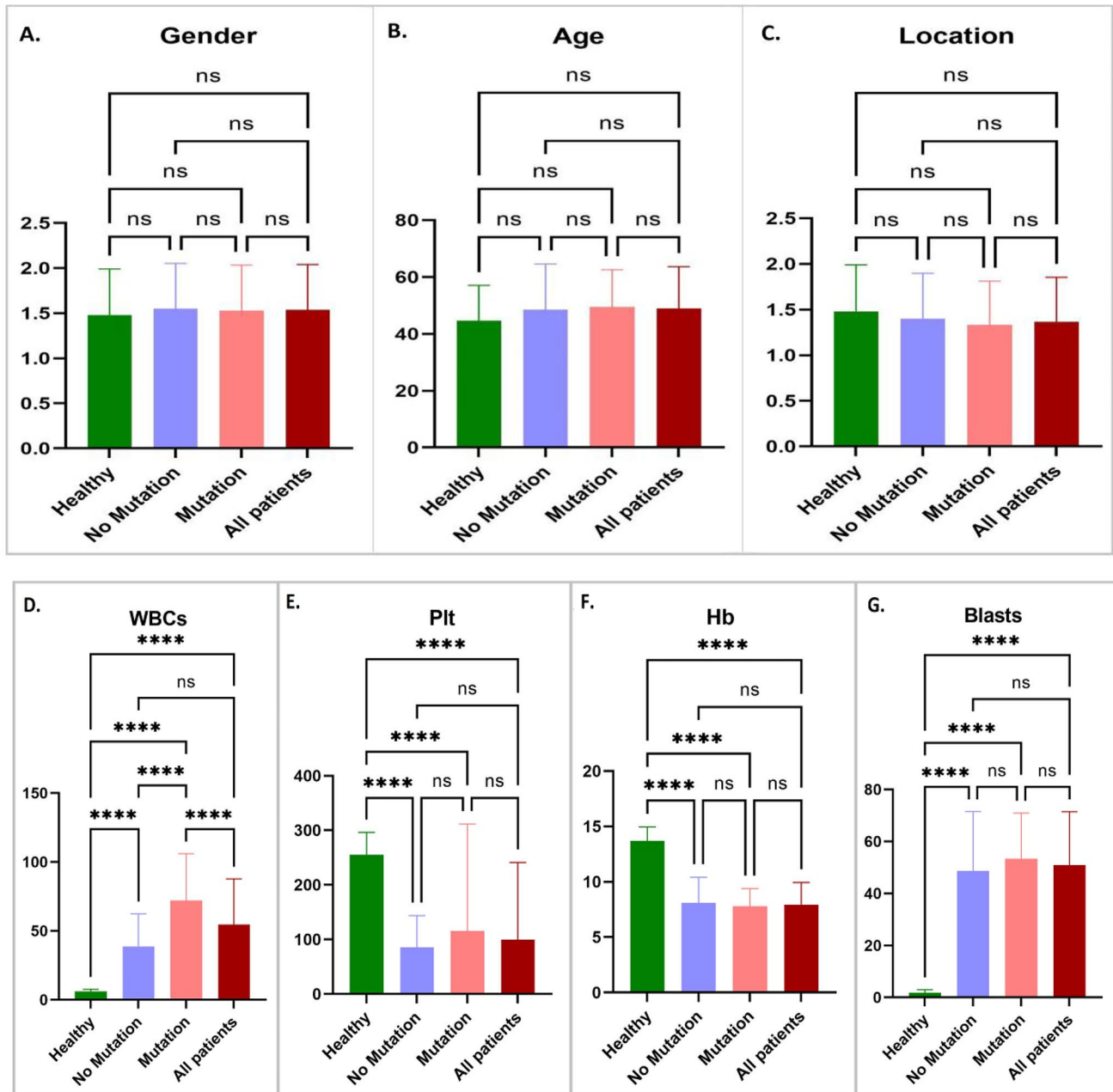
Fig. 5 Presents the differences between AML patients (both mutated and unmutated cases) and a control group in several key aspects: **(a)** gender, **(b)** age, **(c)** location, **(d)** total WBCs, **(E)** platelets **(F)** Hb and **(G)** peripheral blasts

Note: Chi-square significant at $p \leq .05$. p-Value style: GP: >0.05 (ns), 0.0001 (****) [26]

were 34 (85.0%) remissions, 4 (10.0%) failures, and 2 (5.0%) deaths. Significant differences were observed in the outcomes between patients with and without detected mutations (p-value < 0.001) Fig. 7. This suggests that the presence of these specific mutations may have an impact on the response to treatment and overall prognosis in AML patients.

Discussion

In this retrospective cohort study, our aim was to investigate the frequency and prognostic impact of FLT3-ITD, DNMT3A, NPM1, and TP53 mutations in patients with newly diagnosed AML. These genetic mutations play a significant role in disease pathogenesis and are considered important predictors of treatment response and outcomes.

Fig. 6 Performed Pearson correlation and polynomial regression analyses **(A).** Correlations between mutated and unmutated AML patients **(B).** Correlations between AML patients with control group regarding clinical parameters (gender, age, location, total WBCs, Hgb platelets, peripheral blasts and OIT)
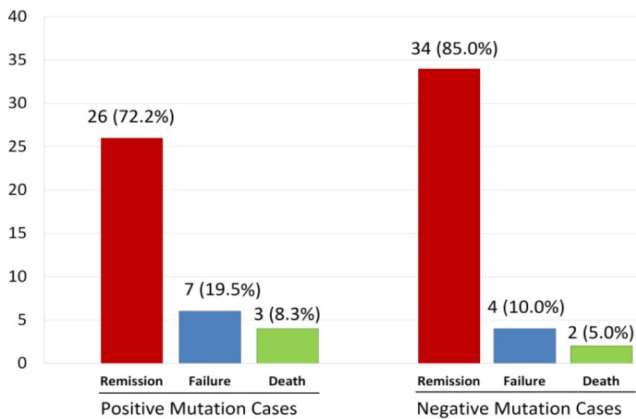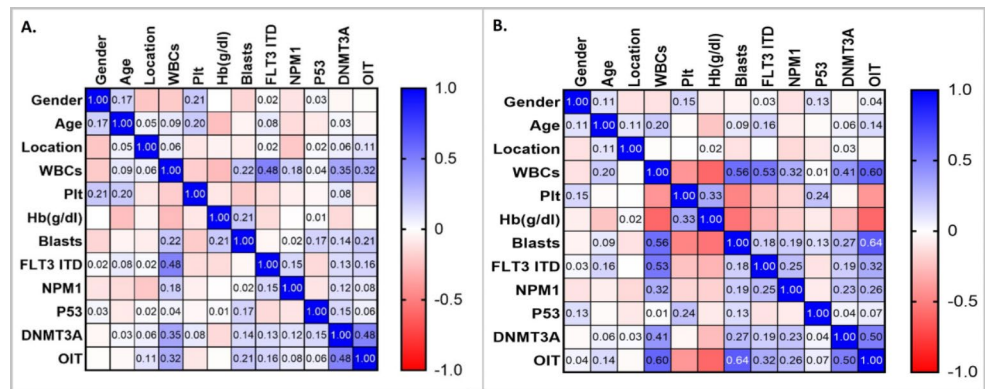

Fig. 7 Clinical Outcome of studied cases after 60 days of complete treatment and induction therapy

To the best of our knowledge, there has been limited evaluation of these mutations in this specific region. Therefore, our study aimed to fill this knowledge gap and provide insights into the prevalence and prognostic implications of these mutations in AML patients. The mutation frequencies observed in our study were as follows: *FLT3-ITD*: 23.7%, *DNMT3A*: 18.4%, *NPM1*: 14.5%, and *TP53*: 6.7%. These mutations were associated with worse outcomes independent of other clinical markers such as age, total WBCs counts, blasts, and response rates during induction therapy.

These findings highlight the importance of genetic investigation in the diagnosis, prognosis, and treatment of AML. Identifying these mutations can provide valuable information for tailoring treatment strategies and predicting patient outcomes [34]. However, the varied results between studies may be due to possible differences in sample size as well as different methods used to evaluate these mutations [35].

The most common genetic abnormalities found in patients with AML are *FLT3* mutations. In fact, *FLT3-ITD* is one of the current molecular genetic markers used for the diagnosis and risk assessment of AML [35]. We found the incidence of *FLT3-ITD* mutations to be 23.7%, which was marginally similar to that found in a Japanese cohort (23%) [36], higher than that found in an Indian cohort (10.8%) [37] and

lower than that in a study from the United Kingdom (27%) [38]. In this study, the first mutation detected (c.1751delc) was previously reported [28], while the other two mutations are reported for the first time, and were deposited in NCBI-GenBank as novel mutations with the accession numbers OP807465 and OP807466. These novel mutations were predicted to be 'disease-causing' according to the mutant tester tools. Both mutations were computationally predicted by I-Mutant software to alter the protein function and decrease protein stability, with predicted DDG values < 0 [25, 39]. Moreover, the VarSite tool showed that amino acid residues for both mutations with a disease propensity value of 1.15 and 1.50, were predicted a disease-associated variants [25]. These computational predictions provide insights into the potential functional consequences of the identified mutations and contribute to our understanding of their impact on the protein's structure and stability in the context of the disease.

The FLT3 was found to be overexpressed in leukemia and to be a prognostic factor for poor prognosis in AML. Patients with high FLT3 expression also have high levels of leukemic oncogenes. As a result, high FLT3 expression is a risk factor for leukaemia [40]. Cases with FLT3-ITD mutations are associated with a higher level of WBCs and high blasts percentage, with lower hemoglobin and platelets [28, 41].

Additionally, the frequency of *NPM1* mutations was 14.5%, as reported previously in India (14.4%) [37]. However, this frequency was lower than that reported in the United Kingdom (21%) [15] and in a separate study in India (27.9%) [8], and higher than that previously reported in Iraq (10%) [31]. A duplication of TCTG at positions 956 to 959 in the reference sequence (c860-863dupTCTG), known as *NPM1* mutation A, accounts for 75–80% of instances [30]. In our study, it accounted for 72.2%. Additionally, another mutation in exon 12 (c868G > T) was found, accounting for 27.3% of *NMP1* mutations, as reported earlier in Iraq [31]. Exon 12 was the location of the majority of examined *NPM1* mutations. Regardless of the mutation, all variations result

in changes at the C-terminus of the NPM leukemic mutants that cause the protein to dislocate into the cytoplasm. These changes include tryptophan(s) 288 and 290 and the creation of an additional nuclear export signal (NES), and this protein has a tendency to be stable and accumulate in the cytoplasm [42].

Furthermore, we observed two missense *TP53* mutations in five patients, c764T > A and c826G > C. Both mutations were reported previously in other studies [32, 33]. They are associated with distinct clinico-biological features and were a poor prognostic factor in this study's AML patients, independent of age, WBC count, karyotype and other genetic markers. Most *TP53* mutations were found in exons 5–8 [11]. *TP53* is noted in 6–14% of AML cases, and in general, *TP53* mutations confer an adverse prognosis with documented chemo-resistance. *TP53* mutations may be mainly responsible for the very poor prognosis of complex karyotype AML [43, 44].

In our previous study, which included the same patient sample along with an additional fifteen AML patients who did not have a *DNMT3A* mutation, we identified three novel DNMT3A mutations. These mutations were found in 14 out of the total number of patients, accounting for approximately 18.4% of the patient population [25]. This result was lower than that obtained in a US cohort (21.9%) [45] and higher than that in a study in China (14.0%) [46]. The mutations that were detected in the DNMT3A gene included heterozygous V877I and L888Q, which were caused by c2629G > A and c2663T > A substitutions, respectively. Additionally, a frameshift mutation c2635delA was identified, resulting in protein truncation with a stop codon at position N879T*. Furthermore, promoter methylation analysis of the DNMT3A gene was performed using the methylation-specific PCR (MSP) technique. The results revealed that promoter methylation was present in 9 out of the total number of patients, accounting for approximately 64.28% of the sample. Importantly, a significant correlation was observed between DNMT3A mutations and promoter methylation (pv = $8.52 \times 10^{\wedge}5$) [25].

Multiple mutations frequently coexist in the same patient, indicating that coordinated interactions between mutations may play a role in the development of AML [47]. The difference in the frequency of these mutations might be due to the geographical distribution of the population studied [37]. In the present study, AML were patients divided into 10 groups based on genetic markers. Interactions of genetic alterations in AML frequently were reported, except the *NPM1* mutation, which was not observed in patients with *TP53* mutations. In a previous study, AML patients with three *FLT3/NPM1/DNMT3A* gene mutations (AML FLT3/NPM1/DNMT3A) presented with a high disease burden and had

an average elevated WBC ($> 100.000 \times 10^{+9}$/L) compared to other groups [48].

The clinico-haematological characteristics observed in this study are consistent with findings from other studies conducted globally. The study examined the relationships and correlations between gene mutation status and parameters such as Hb, total WBCs, PLT, location, and blasts percentage. It is worth noting that age and gender did not exhibit statistically significant differences between the mutation and non-mutation groups. This finding is in line with a previous study that also reported no difference in mutation frequency between males and females [49]. However, mutated and unmutated patients had elevated blast cells and low platelets and haemoglobin counts, with statistically significant differences and correlations were reported in total WBC count in patients with gene mutations compared with unmutated patients [41]. Indeed, elevated WBCs can be caused by a variety of conditions, including infections and autoimmune disorders. Furthermore, the presence of blasts in the blood is the best indicator of acute leukaemia. Blasts are cancerous cells that are still immature but replicate rapidly, causing tiredness from anemia and a risk of bleeding from a low platelet count. They slow down the production of red blood cells and platelets [50]. However, current study reported a significant differentiation and correlations in AML cases compared to a control group enrolled in the genetic and hemato-pathological investigations (Fig. 5A and B).

Importantly, we found significant correlations between patients with detected mutations and patients without mutations in disease progression after clinical induction therapy (remission, failure and death; p = < 0.001) [51]. These correlations indicate that low responses to treatment and higher mortality rates after 60 days of induction therapy (Fig. 6). The general approach for therapy has not substantially changed in recent years [44]. Intensive clinical induction therapy is conducted with 3 days of an anthracycline and 7 days of cytarabine (commonly referred to as '713' regimens) that aims at killing off all leukemic cells and forcing the patient into remission. Sometimes several rounds of induction with increasing potency must be undertaken in order to obtain the desired result. Complete remission (CR) is achieved in 60–80% of younger adults and in 40–60% of older adults (60 years or above) [1]. In addition to their function in the pathogenesis of the disease, molecular mutations in AML are considered important predictors of patients' response to therapy and outcomes [37]. Efforts to develop protein kinase inhibitors, that target mutated forms of the FLT3 receptor, have led to successive generations of FLT3 inhibitors [44]. FLT3 inhibitors have received FDA approval and have demonstrated increased clinical response in AML patients when used alone or in combination with chemotherapy [52]. Therefore, the European Leukaemia

Network (ELN) recomended that all AML patients have access to *FLT3* mutational test data within 72 h to guide treatment decisions based on the patient's mutation status [44]. Every AML subtype described under the heading 'AML with recurrent genetic abnormalities' has a specific genetic mutation that must be identified to determine prognosis and guide treatment. For example when there is no concurrent *FLT3-ITD* mutation, *NPM1* mutations consistently indicate a favorable prognosis in AML patients under 60 years of age; however, in AML patients 70 years of age or older, *NPM1* mutations seem to be the sole factor influencing prognosis in multivariate analysis [16]. Patients with *NPM1*-mutated AML who have high *FLT3-ITD* (ratio ≥ 0.5) should receive conventional chemotherapy as well as a FLT3 inhibitor (which was not approved at the time our patients were treated). These patients may benefit from the incorporation of GO into frontline therapy [53].

In patients with AML, the treatment is usually divided into two chemotherapy phases: remission induction and consolidation. Remission induction aims to achieve a complete remission by reducing the number of leukemic cells. Consolidation therapy is then given to eliminate any remaining leukemic cells and lower the risk of relapse. Currently, it was observed that the presence of mutations had an impact on the remission rate in AML patients. Certain genetic alterations were found to influence the response to treatment and the achievement of remission, death often occurs due to complications of marrow failure, such as sepsis or significant hemorrhage. Furthermore, undiagnosed, rapidly evolving cases may present with fatal multi-organ failure [54, 55].

Conclusion

The study highlights that FLT3-ITD, DNMT3A, and NPM1 are frequently observed genetic alterations in Iraqi adult patients with AML. Molecular testing at various stages of the disease, including diagnosis, remission, and relapse, can provide valuable data to guide personalized clinical therapy. These genetic panels generate extensive data beyond the current diagnostic and prognostic criteria for AML patients. As our understanding of the impact of somatic mutations and their complex interactions continues to improve, this information may be utilized in the future to optimize and individualize treatment strategies, increasing the likelihood of achieving complete remission. It is therefore crucial to preserve AML patient samples in biobanks for future genomics and proteomics analyses, enabling the exploration of mutational patterns and the development of new targeted therapies.

Declarations

Ethics approval and consent to participate The ethical aspects of this study were given utmost importance and followed the established guidelines and regulations. Prior to enrollment, all patients provided verbal consent for the collection of blood samples. The study protocol was reviewed and approved by the Ministry of Health's health directorate, with the approval obtained on August 23, 2021 (protocol NO. 9811). The research investigations were conducted at the University of Sulaimani, College of Science, in accordance with the applicable rules and regulations. The university granted approval for the study (No. 1917/259 on 01/08/2021), ensuring that ethical standards were upheld and necessary protocols were followed throughout the study. The study was conducted in compliance with the research ethical guidelines set by the Ministry of Health. The ethical standards outlined in the Declaration of Helsinki, which governs medical research involving human subjects, were strictly followed. Participants, including the parents of patients under the age of 18, provided both verbal and written consent after being provided with the necessary information and having the opportunity to review the Declaration of Helsinki.

Consent to participant Not Applicable.

Consent for publication Not Applicable.

Competing interests The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Conflict of interest The authors declare no conflict of interest.

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